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Inhibition of neutrophil superoxide production by human plasma α_1-antitrypsin

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We report here that human plasma α_1-antitrypsin (α_1-AT) inhibited human neutrophil $O_2^{\cdot-}$ release elicited by a variety of stimulants. In comparison, the inhibitory capacities of two serine protease inhibitors, L-1-tosylamide 2-phenylethyl chloromethyl ketone (TPCK) and soybean trypsin inhibitor (SBTI), and the human recombinant α_1-AT mutant, α_1-AT-Arg^{358} were in the order: α_1-AT = TPCK ≫ α_1-AT-Arg^{358} > SBTI when cells were stimulated with concanavalin A plus cytochalasin E. These data suggest that, in human inflammatory fluids containing relatively high concentrations of α_1-AT (such as rheumatoid arthritis synovial fluid), (i) α_1-AT may down-regulate the inflammatory process by inhibiting the neutrophil respiratory burst and (ii) serpin oxidation by neutrophil-released reactive oxygen species is unlikely to occur.

α_1-Antitrypsin; Serine protease inhibitor; Neutrophil respiratory burst; Superoxide anion radical; Inflammation; Human

1. INTRODUCTION

Human plasma α_1-antitrypsin (α_1-AT) is an acute phase protein, which is synthesized in increased amounts by the liver during inflammation, and is a member of the serpin (serine protease inhibitor) superfamily of proteins (reviewed in [1]). Although α_1-AT is an inhibitor of both trypsin and neutrophil elastase, neutrophil elastase is the physiological target for α_1-AT. The release of neutrophil elastase has been implicated in the tissue damage associated with chronic inflammatory diseases such as pulmonary emphysema and rheumatoid arthritis [2]. Cigarette smoking is a major risk factor for pulmonary emphysema [1] and bronchoalveolar lavage fluids from smokers, along with knee-joint synovial fluids from rheumatoid arthritis patients, show a reduction in anti-elastolytic activity compared with that expected from the α_1-AT concentration [3–5], containing oxidised and proteolytically fragmented forms of α_1-AT [2,6,7].

Exposure of neutrophils to a variety of stimuli (including concanavalin A plus cytochalasin E) activates a membrane-bound NADPH-oxidase to catalyse the generation of the superoxide anion radical ($O_2^{\cdot-}$) (reviewed in [8]). This radical species dismutes to H_2O_2 which is in turn converted to hypochlorous acid (HOCl) in a reaction catalysed by neutrophil myeloperoxidase [8]. In vitro experiments have suggested that the mechanism of α_1-AT inactivation involves oxidation of the reactive centre (Met^{358}) residue by neutrophil- or macrophage-derived oxidants, such as HOCl [8,9]. However, the proteolytic inactivation of α_1-AT by neutrophil collagenase [8,10,11], as well as connective tissue metalloproteinases [12–14], may also play a role in the inactivation process.

During the course of experiments designed to study the mechanisms of α_1-AT inactivation by isolated human neutrophils, we noted that the amount of detectable $O_2^{\cdot-}$ released by cells stimulated with a variety of agents (serum-treated zymosan, calcium ionophore or concanavalin A (Con A) plus cytochalasin E (Cyto E)) was lowered in the presence of α_1-AT. Earlier studies, which were largely performed with Con A + Cyto E as the stimulus, showed that L-1-tosylamide 2-phenylethyl chloromethyl ketone (TPCK), soybean trypsin inhibitor (SBTI) and other serine protease inhibitors have the ability to inhibit $O_2^{\cdot-}$ production by neutrophils ([15] and references therein) and by other cell types [15,16]. Therefore, the dose-dependent inhibition by α_1-AT of Con A + Cyto E-stimulated neutrophil $O_2^{\cdot-}$ production was compared with the dose responses for TPCK, SBTI and α_1-AT-Arg^{358}. The latter is a human recombinant α_1-AT variant in which the reactive centre Met^{358} has been replaced by an arginine residue [17]. This single amino acid substitution results in a 10,000-fold decrease in anti-elastolytic activity and a 10,000-fold increase in thrombin inhibitory capacity.

Abbreviations: α_1-AT, α_1-antitrypsin; TPCK, L-1-tosylamide 2-phenylethyl chloromethyl ketone; SBTI, soybean trypsin inhibitor; Con A, concanavalin A; Cyto E, cytochalasin E.

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2. MATERIALS AND METHODS

Concanavalin A (Type IV-S), cytochalasin E (from *Aspergillus clavatus*), cytochrome *c* (Type III, from horse heart), trypsin inhibitor (Type I-S, from soybean), superoxide dismutase (from bovine erythrocytes), L-1-tosylamide 2-phenylethyl chloromethyl ketone, calcium ionophore A 23187, zymosan A, *N*-succinyl-Ala-Ala-Ala *p*-nitroanilide and elastase (Type IV, from porcine pancreas, 120 U/mg protein) were purchased from Sigma (Poole, Dorset, UK). Human plasma α_1-AT was obtained from Novabiochem (Nottingham, UK). The purity of this preparation was >95% by SDS-PAGE. The elastase inhibitory capacity of the α_1-AT batches used in these experiments was assessed by a kinetic spectrophotometric determination of the ability of α_1-AT to inhibit the cleavage of a synthetic substrate for porcine pancreatic elastase [18]. The anti-elastolytic activity of the two batches was 1.06 ± 0.10 and 1.18 ± 0.08 (mean ± 1 S.D., n = 5) mol of α_1-AT required to inhibit 1 mol of elastase. Recombinant α_1-AT-Arg^{358}, expressed in yeast, was kindly provided by Dr. Michael Courtney, Delta Biotechnology Ltd., Nottingham, UK.

Protein concentrations were determined by the Bradford assay [19]. TPCK, cytochalasin E and A 23187 were dissolved in dimethyl sulphoxide and diluted with Hanks' balanced salt solution (Gibco, Uxbridge, Middlesex, UK) immediately before use. Serum-treated zymosan was prepared according to Goldstein et al. [20].

2.1. *Preparation of cells*

Human peripheral blood neutrophils were isolated from fresh heparinised blood obtained from healthy adult volunteers by dextran sedimentation followed by density gradient centrifugation and hypotonic lysis of the remaining red blood cells [21]. Finally, the cells were resuspended in Hanks' balanced salt solution without Phenol red, counted and diluted to a concentration of 5×10^6 cells/ml. Cell viability was assessed by Trypan blue exclusion.

2.2. *Measurement of $O_2^{\cdot-}$ production*

Superoxide anion production was measured by superoxide dismutase inhibitable reduction of cytochrome *c* using a recording double beam spectrophotometer (Uvikon 860, Kontron Instruments, Watford, UK) equipped with a thermostated 6-cuvette holder.

The cells (5×10^5) were pre-incubated for 3 min at 37°C with various amounts of inhibitors (TPCK, SBTI, α_1-AT, α_1-AT-Arg^{358}) in Hanks' (final volume, 700 μl). Cytochrome *c* (100 μl; final concentration, 100 μM) was added to both sample and reference cuvettes and in the sample cuvettes the neutrophils were stimulated with Con A + Cyto E (200 μl + 100 μl; final concentrations, 100 and 10 μM, respectively), serum-treated zymosan (2 mg/ml, final concentration) or A23187 (30 μM, final concentration). Both sample and reference cuvettes contained the same concentrations of cells and dimethyl sulphoxide. The basal release of $O_2^{\cdot-}$ was automatically subtracted and the absorbance changes recorded were due solely to the Con A + Cyto E-induced respiratory burst. The reduction of cytochrome *c* was monitored at 550 nm at 1 min intervals for 15 min at 37°C. Six samples were monitored simultaneously, one acting as a positive control (cells + stimulatory agents only) and one as a negative control (cells + stimulatory agents + superoxide dismutase).

The amount of $O_2^{\cdot-}$ produced (nmol $O_2^{\cdot-}$/10^6 cells) was calculated using an extinction coefficient of 21×10^3 M^{-1}·cm^{-1} and plotted as a function of time after addition of stimulants to obtain the initial rate of each reaction. The inhibitory capacity was calculated as the % inhibition of the initial rate of the positive control.

Table I

Inhibitory effect of α_1-AT, TPCK and SBTI on neutrophil $O_2^{\cdot-}$ release elicited by various stimulants

Inhibitor	Con A + Cyto E[a]	% Inhibition Serum-treated zymosan[b]	A 23187[b]
α_1-AT (11.5 μM)	98.1 ± 2.8	62.0	67.1
TPCK (10 μM)	96.2 ± 6.7	28.7	90.2
SBTI (500 μM)	94.9 ± 4.1	61.6	92.8

[a] Results are expressed as the mean value ± 1 S.D. (n = 5)
[b] Results are expressed as the mean value (n = 2)

3. RESULTS

α_1-AT, TPCK and SBTI showed various degrees of inhibition of the neutrophil $O_2^{\cdot-}$ production elicited by serum-treated zymosan, calcium ionophore A 23187 and Con A plus Cyto E (Table I). Whatever the triggering agent, significant amounts of inhibition were obtained with both α_1AT and TPCK at similar molar concentrations (11.5 and 10.0 μM, respectively). However, for SBTI, the molar concentration required to give similar extents of inhibition was about 50-fold higher (Table I).

α_1-AT at a concentration of 0.6 mg/ml (11.5 μM) almost completely inhibited $O_2^{\cdot-}$ release from human neutrophils stimulated with Con A + Cyto E. Fig. 1 shows the results of a typical experiment. The inhibition was dose-dependent and was still significant at 0.2 mg/ml (3.8 μM).

α_1-AT-Arg^{358} showed no effect over the effective dose range of α_1-AT. At high concentrations α_1-AT-Arg^{358} produced some inhibition (115 μM gave a 65% inhibition) (Fig. 2). Two serine protease inhibitors (TPCK and SBTI) tested showed dose–response effects similar to those previously obtained by Kitagawa and coworkers [15] (Fig. 2). The concentration range used was 0.2–10 μM for TPCK and 100–500 μM for SBTI. Table II shows the K_i values (concentration required for 50% inhibition of $O_2^{\cdot-}$ production) for the four serine protease inhibitors. α_1-antitrypsin has a K_i lower than SBTI and similar to TPCK.

The serine protease inhibitors tested had no cytotoxic

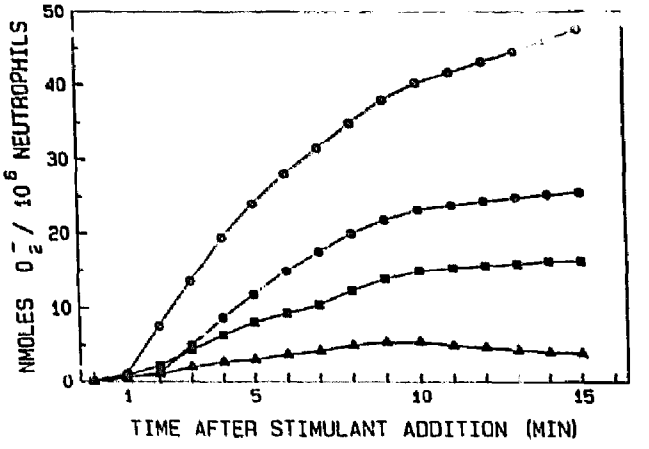

Fig. 1. Time-course of the inhibitory effect of α_1-antitrypsin on $O_2^{\cdot-}$ production by stimulated human neutrophils. (○) Con A + Cyto E alone; (●) 3.8 μM α_1-AT; (■) 5.7 μM α_1-AT; (▲) 11.5 μM α_1-AT.

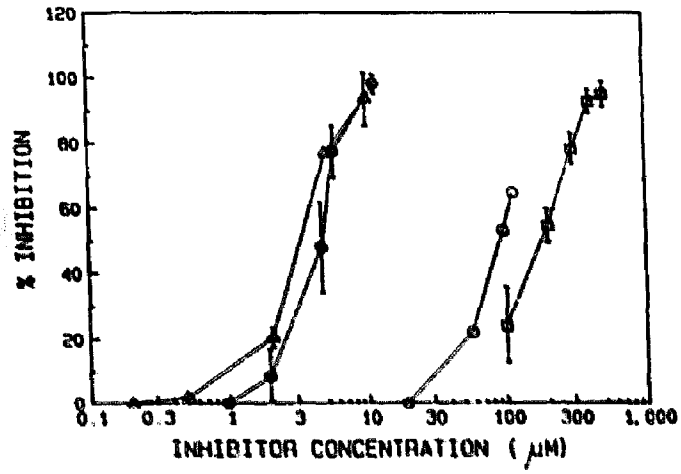

Fig. 2. Dose-response curves showing the inhibitory effect of serine protease inhibitors on $O_2^{\cdot-}$ production by stimulated(Con A + Cyto E) human neutrophils. (○) TPCK; (●) α_1-AT; (▲) α_1-AT-Arg^{358}; (□) SBTI. Each data point represents the mean value of 2–5 experiments (± 1 S.D. where $n \geq 3$).

effect as assessed by Trypan blue exclusion tests performed at the end of activation experiments. In addition, at the concentrations used in the neutrophil experiments, none of these inhibitors possessed $O_2^{\cdot-}$ scavenging activity in a xanthine-xanthine oxidase system coupled with the cytochrome *c* assay (data not shown), which also shows that the α_1-AT preparation was not contaminated with superoxide dismutase.

4. DISCUSSION

We have shown that at sub-physiological concentrations α_1-AT inhibits the oxidative burst of stimulated neutrophils. For comparison, the concentration of α_1-AT in both normal human plasma and knee-joint synovial fluid from rheumatoid arthritis patients is about 3–5 mg/ml, although on average about 40% of the α_1-AT in rheumatoid arthritis synovial fluid is inactive [5].

α_1-AT-Arg^{358}, which has relatively weak anti-elastase activity compared with α_1-AT, only inhibited neutrophil $O_2^{\cdot-}$ production at high concentrations, suggesting that this effect was related to protease inhibitory capacity. This interpretation is further sustained by the lack of $O_2^{\cdot-}$ scavenging capacity of all the inhibitors tested. In agreement with previous results [15], serine protease inhibitors which are not members of the serpin protein superfamily also inhibited neutrophil $O_2^{\cdot-}$ production. The high molecular weight inhibitor of trypsin (SBTI) and the low molecular weight inhibitor of chymotrypsin (TPCK) act by inhibiting a neutrophil plasma membrane trypsin/chymotrypsin-like protease [15].

Recently, Kilpatrick et al. [22] reported the inhibition of human neutrophil $O_2^{\cdot-}$ generation by α_1-antichymotrypsin. These workers mention that a commercial preparation of α_1-AT (Sigma) had no inhibitory effect at concentrations ranging from 0.1 to 100 μM. This discrepancy might be accounted for by a difference in the activities of the α_1-AT preparation used in this study and that used by Kilpatrick and co-workers.

In many of the earlier studies in which neutrophil/macrophage-mediated oxidative inactivation of α_1-AT was demonstrated in vitro (for example [9,23]), the concentrations of α_1-AT would have been too low to significantly inhibit $O_2^{\cdot-}$ production. The α_1-AT concentrations used in these experiments reflected the concentration range in bronchoalveolar lavage fluids (up to 100 μg/ml). However, in some inflammatory exudates the α_1-AT concentration is much higher, e.g. rheumatoid arthritis synovial fluid. In experiments where neutrophils were incubated with α_1-AT at concentrations in this higher range, the α_1-AT was either not in direct contact with the cells, e.g. compartmentalised in dialysis tubing in the presence of external, stimulated neutrophils [24] or, the α_1-AT was inactivated by the addition of a supernatant which had been conditioned by stimulated neutrophils [11,25].

We conclude that, in inflammatory fluids containing relatively high concentrations of α_1-AT, this serpin might play a physiological role in directly inhibiting neutrophil $O_2^{\cdot-}$ production. Although it might be argued that the stimulus mainly used in this study (Con A + Cyto E) is not physiologically relevant, other studies (see references cited in [15]) as well as our own results obtained with serum-treated zymosan and calcium ionophore show that serine protease inhibitors inhibit neutrophil $O_2^{\cdot-}$ production in response to a variety of stimuli. Elastase has been shown to prime neutrophil $O_2^{\cdot-}$ release elicited by different stimulants [26,27]. Our experimental system contained no added elastase to prime the system prior to stimulant addition, suggesting that α_1-AT inhibited neutrophil $O_2^{\cdot-}$ release by inhibiting a membrane bound serine protease. However, in vivo, inhibition of elastase may be an additional mechanism by which α_1-AT down-regulates the neutrophil oxidative response.

Our results also suggest that, in those inflammatory fluids where the α_1-AT concentration is relatively high, the oxidation of α_1-AT by neutrophil-released reactive oxygen species is unlikely to occur. However, other cell types may mediate the oxidation of α_1-AT and it is

Table II

K_i (concentration required for 50% inhibition of $O_2^{\cdot-}$ production) of serine protease inhibitors in Con A + Cyto E-triggered $O_2^{\cdot-}$ release by human neutrophils. Values were calculated form Fig. 2.

Serine protease inhibitor	K_i (μM)
α_1-AT	5.1 (μM)
α_1-AT-Arg^{358}	93.6 (μM)
TPCK	4.7 (μM)
SBTI	207.0 (μM)

possible that neutrophil metalloproteinases inactivate α_1-AT locally, thus permitting elastase activity.

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